VIP Antitumor Vaccines

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Synthetic Vaccines of Tumor-Associated Glycopeptide Antigens by Immune-Compatible Thioether Linkage to Bovine Serum Albumin**

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Dedicated to Professor Peter Welzel on the occasion of his 70th birthday

The mucin MUC1 is expressed in normal cells only on the apical surface. In epithelial tumor cells, however, it is strongly over-expressed all over the cell surface. In carcinoma cells, characteristically altered saccharide side chains are found on this glycoprotein as a result of the deficient activity of glycosyl transferases.[1] A distinctly decreased expression[2] of β-1,6-*N*-acetylglucosaminetransferase C2GnT-1 reduces the formation of the core-2-saccharide structure from which the long polylactosamine saccharide chains typical for normal epithelial cells are assembled. Furthermore, an up to tenfold increased activity of sialyltransferases[3] in tumor cells results in early sialylation of premature T_N- and T-antigen structures[4] to give the important tumor-associated sialyl-T_N-, 2,3- and 2,6-sialyl-T-antigen structures,[5–8] and they are thus withdrawn from any further glycan assembly.

In order to utilize these tumor-associated saccharide antigens for the induction of antibodies, such saccharides have been linked to carrier proteins. A number of conjugation methods particularly important for the weakly immunogenic carbohydrate antigens have been developed for these purposes.[10] Recently introduced conjugation procedures include oxime formation from carbonyl groups,[11] reactions of thiols with iodoacetamides,[12] formation of disulfides,[13] addition of thiol groups to maleimides,[14] Staudinger reaction of azides combined with ester aminolysis,[15] 1,3-dipolar cycloaddition of azides[16] to alkynes,[17] and the aminolysis of squarates.[18]

In comparison to the conjugation of saccharides, the coupling of glycopeptide antigens to proteins proves more problematic because of the additional functional groups. If the glycopeptide includes no further carboxylic functions apart from the C-terminal group, conjugation to, for example, bovine serum albumin can be achieved by active ester formation in water.[19] If sialic acid residues and further carboxylic groups are present in the glycopeptide, these functions cannot be protected since their deprotection is impossible after conjugation to the protein. Using amino-functionalized spacers and based on the graduated reactivity of squarates,[18] the coupling of sialyl-T_N-antigen glycopeptides to BSA was accomplished to result in synthetic vaccines.[20] Heterobifunctional linkers, for example, *N*-succinimidyl-4-(maleimidomethyl)cyclohexane carboxylate (SMCC)[21] have been used for conjugation by Michael addition of SH groups.

The immune response induced by this type of vaccines is of value for diagnostic purposes as long as monoclonal antibodies selectively binding to tumor cells can be obtained by selection and cloning.[22] However, the utilization of the selective immune response against tumor-associated glycopeptide antigens in an immunotherapy might be prevented because the thiosuccinimides formed from the maleimides, as well as the squaric acid and (hetero)aromatic linker structures, are immunogenic themselves, and the immune response towards the glycopeptide hapten can be strongly suppressed.[23] Therefore, the development of antitumor vaccines requires nonimmunogenic linkers. As the specificity of the glycopeptide antigens obviously depends upon their conformation,[24] such linker structures should contain as few hydrogen-bond donors and acceptors as possible but should still promote water solubility. Alkyl thioethers are expected to fulfill these requirements.

The formation of thioethers through photochemical, that is, radical-induced, addition of thiols to alkenes[25] has already been applied for the linkage of carbohydrates to alkenes.[26] In order to clarify whether this reaction applies to the conjugation of amino acids and peptides, corresponding transformations of model compounds were performed. As shown in Scheme 1, the photoinduced radical-type thioether formation proceeded with stoichiometric amounts of reactants in water (**3a**), water/methanol (**3c**,**d**,**f**,**g**), water/ethanol (**3e**), and methanol (**3b**) at room temperature. With the radical starter VA-044, the reaction was thermally induced (**3c**,**d**,**f**). Unreacted alkene components were recovered. The use of an excess of thiol resulted in a complete conversion of the allylic component. As a rule, the disulfide of the cysteine derivative is the only side product (approximately 10%). In contrast to reports in the literature,[27] the α position of the amino acids is not affected. Tyrosine (**3b**) and tryptophan derivatives (**3c**) susceptible to oxidation, and polar amino acid residues (**3a**,**d**) present in the tandem repeat domain of MUC1 are not affected. In addition, this coupling reaction can be used for the linkage of spacer molecules (**3e**), fluorescence labels (**3f**), and biotin markers (**3g**). The thioether linkage can be achieved by irradiation alone or with the help of a water-soluble radical initiator (ACVA orVA-044). The addition of the radical initiator (5–25 mol%)

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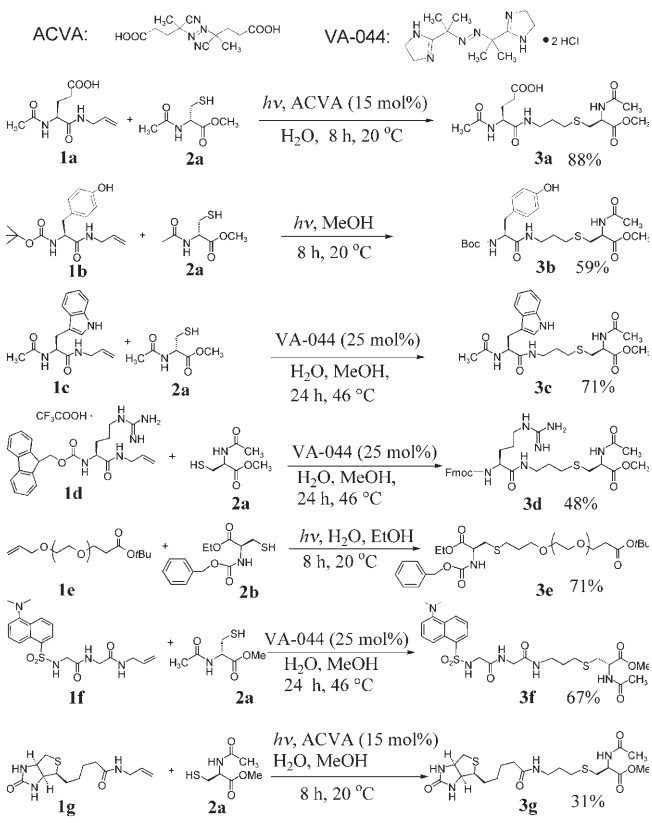
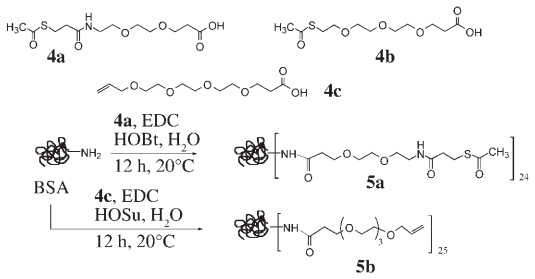


Scheme 1. Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl. The indicated yields refer to purified compounds.

increased the yield by about 10% within the same reaction time.

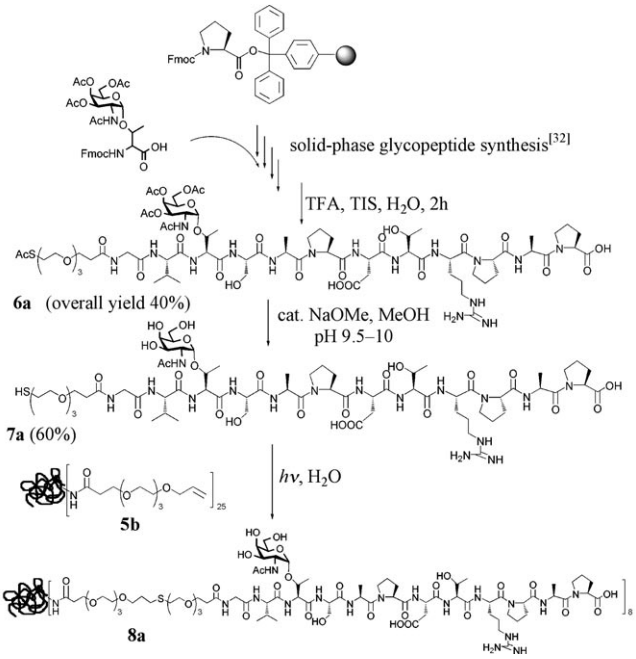
As antigenicity and specificity of glycopeptides apparently depend upon their conformation,[24,28] it is advisable for the conjugation of such haptens to proteins to insert hydrophilic spacers. Heterobifunctional spacers **4** (Scheme 2) were synthesized from di- and triethylene glycol according to previously described procedures.[29] They can be used in two approaches for the immune-compatible conjugation of glycopeptide antigens, by linking either the thiol component or the alkene component to the carrier protein BSA.

The coupling of the S-protected thiol spacer **4a** (200 equiv) to BSA was performed in water at room temperature under weakly acidic conditions using EDC in the presence of HOBt or HOSu.[19] The conjugate **5a** was purified by dialysis against water. Besides the terminal amino group, BSA contains 59 lysine residues of which only 30 are easily accessible. The MALDI-TOF analysis of the conjugate **5a** revealed that it contains 24 spacer molecules per molecule of BSA on average. The coupling of olefinic linker **4c** to BSA (Scheme 2) gave **5b** loaded on average with 25 linker molecules per molecule of protein according to MALDI-TOF mass spectrometry.



Scheme 2. EDC = *N*-(dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole, HOSu = *N*-hydroxysuccinimide.

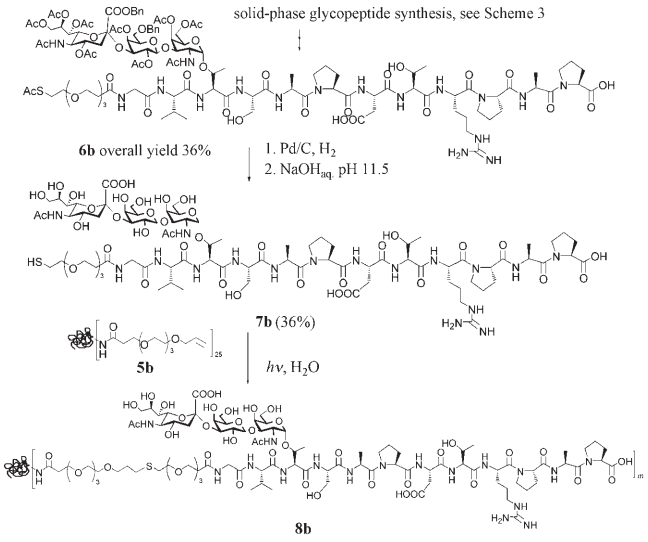
The BSA derivatives **5** decorated with the linker molecules were then used for the synthesis of the thioether-linked BSA–glycopeptide vaccines according to the two alternative procedures (linkage of either the thiol component or the alkene component to the carrier protein). Glycopeptide antigens of MUC1 provided for coupling with BSA **5b** modified with the olefinic linker were synthesized using the trityl linker[30] on Tentagel resin[31] (shown in Scheme 3 for the T_N antigen glycopeptide) as previously described.[32] At the end of the solid-phase synthesis the N-terminal glycine was acylated with the heterobifunctional linker **4b**, and the glycopeptide was detached from the resin by simultaneous cleavage of the trityl linker and all acid-labile side-chain protecting groups.



Scheme 3. TFA = trifluoroacetic acid, TIS = triisopropylsilane.

The S- and O-acetyl groups of the product **6a** (overall yield relative to loaded resin: 40%) were removed with cat. sodium methoxide in methanol. After purification by semi-preparative HPLC, the thiol-terminated glycopeptide antigen **7a** was coupled to the olefin-modified BSA **5b** in water by irradiation.[34] After dialysis and lyophilisation, MALDI-TOF analysis indicated that the antigen–BSA conjugate **8a** contained on average eight molecules of the glycopeptide per molecule of BSA (Scheme 3).

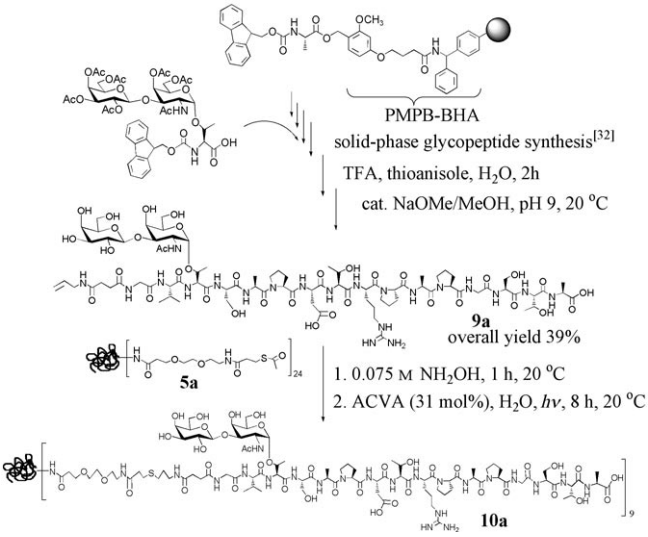
The analogous construction of the 2,3-sialyl-T-antigen MUC1 glycopeptide **6b**[35] containing the protected thiol spacer was achieved using the Fmoc-2,3-sialyl-T-threonine building block.[32b] It is noteworthy that after acidolytic release of **6b** from the resin, the hydrogenolysis of the benzyl ester and benzyl ether of the saccharide moiety was not affected by the thioester functionality. After base-catalyzed transesterification in methanol, the MUC1 glycopeptide antigen **7b** modified with the thiol spacer was subjected to the photochemically induced thioether conjugation to give the synthetic vaccine **8b** (Scheme 4).



Scheme 4.

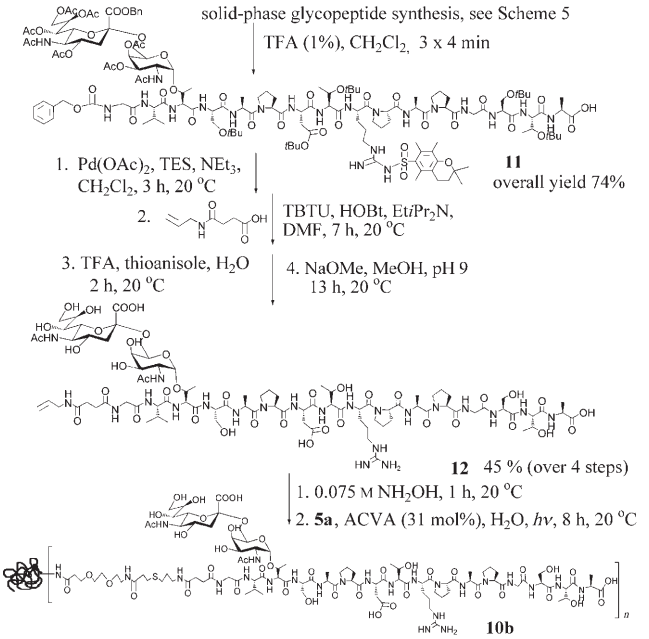
Glycopeptides used in the reverse formation of the thioether linkage were constructed on a polystyrene resin functionalized with benzhydrylamide (BHA) and the super-acid-labile[36] 4-hydroxymethyl-3-methoxyphenoxybutyric acid (HMPB) anchor loaded with the C-terminal amino acid. The glycohexadecapeptide of the MUC1 tandem repeat containing the T-antigen side chain was synthesizd using the T-antigen-threonine building block.[32a] After completion of the solid-hase synthesis, N-terminal acylation with succinic mono-*N*-allylamide was followed by acidolytic cleavage from the resin and Zemplén transesterification (pH 9) to afford MUC1 glycopeptide **9a** equipped with the olefinic spacer (Scheme 5).

Prior to the photochemical linking, BSA **5a** carrying the S-acetylthio spacer had to be deacetylated under argon using 0.075 M aqueous hydroxylamine solution. After dialysis under argon, the thiol groups of the modified BSA were reacted with the allylamide groups of glycopeptide **9a** by irradiation in the presence of the radical starter ACVA. The obtained thioether-linked vaccine **10a** showed only weak signals in the MALDI-TOF mass spectrum. Therefore, its loading was determined photometrically after reaction with phenol/sulfuric acid;[19] the synthetic MUC1 vaccine was found to contain on average nine molecules of the glycohexadecapeptide per molecule protein.



Scheme 5.

The synthesis of a glycopeptide vaccine containing a sialic acid residue required a modified procedure, because the allylamide would be hydrogenated during the hydrogenolysis of the sialic acid benzyl ester. Therefore, a benzyloxycarbonyl(Z)-protected sialyl-T_N-glycopeptide **11** was synthesized on solid phase and then cleaved selectively from the SASRIN anchor (1% TFA in CH_2Cl_2) (Scheme 6). After hydrogenolysis of both the benzyl ester and the Z group, the sialylated glycopeptide was coupled with succinic monoallylamide in solution. Only then were the remaining protecting groups removed by acidolysis and transesterification to give the sialyl-T_N-MUC1-glycohexadecapeptide **12**.[37] Removal of the S-acetyl group from the modified BSA **5a** using dilute



Scheme 6. TES = triethylsilyl, TBTU = benzotriazolyltetramethyluronium tetrafluoroborate.

aqueous hydroxylamine and subsequent photochemical conjugation with glycopeptide **12** furnished the synthetic vaccine **10b**.

The results give evidence that synthetic glycopeptides containing tumor-typical structures in the saccharide and in the peptide portions can be coupled in a structurally exactly specified form to carrier proteins by photochemically induced addition of thiols to alkenes. Side reactions at the α-CH groups of amino acids are not observed. The immune-compatible thioether linkage is achieved by loading the protein with alkene linker/spacer molecules on the one hand and equipping the glycopeptide with a structure terminating in a thiol on the other hand and subjecting both components to the photochemical thioether formation. Alternatively, the protein is decorated with thiol side chains and photochemically conjugated with the glycopeptide antigen bearing olefinic end groups. In the latter procedure, in particular, exclusion of oxygen is necessary. Sialic acid containing glycopeptide protein conjugates show problems in MALDI-TOF mass spectrometry.

The conjugates of synthetic glycopeptides with carrier proteins described herein constitute most versatile forms of synthetic vaccines. After processing of the protein, they provide the T cell epitopes required for activation of T cells,[38] and owing to their ability to adhere to the microtiter plates they are useful for probing the induced sera.[20,22] The non-immunogenic thioether linker formed by means of oligoethylene glycol spacers should open up the opportunity to utilize these advantages in the development of vaccines for active immunization against tumor cells.

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[33] $[\alpha]_D^{23} = -84.6\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 0.02\ \mathrm{g\,cm^{-3}}$, H_2O); HR-ESI-MS (positive): *m/z* 1759.7931 $[M+H]^+$, calcd 1759.7948. ^{1}H NMR (400 MHz, D_2O, COSY): $\delta = 5.29$ (d, 1H, $J_{4,3} = 2.9$ Hz, H-4), 5.09 (dd, 1H, $J_{3,2} = 11.2$ Hz, H-3), 4.99 (d, 1H, $J_{1,2} = 3.7$ Hz, H-1), 2.98 (t, 2H, $J = 6.8$ Hz, S-CH_2), 2.84 (dd, 1H, $J_{gem} = 17.1$ Hz, $J_{vic} = 6.5$ Hz, D_a^{β}), 2.79 (dd, ^{1}H, $J_{vic} = 6.8$ Hz, D_b^{β}), 1.18 (d, 3H, $J = 6.3$ Hz, T^{γ}), 1.06 ppm (d, 3H, $J = 6.4$ Hz, T^{γ}).
[34] Experimental: In a quartz test tube (NS 14.5) **7a** (11 mg, 6.9 μmol) was dissolved in degassed aqueous phosphate buffer (pH 7). Under argon atmosphere, 4 mg of olefin-modified BSA **5b** was added. The solution was irradiated with a mercury low-pressure vapor lamp ($\lambda = 254$ nm, 77 W) for 6 h, dialysed against

distilled water (Spectra/Por Float A Lyzer), and lyophilised to give 7 mg of **8a** as a colorless lyophilisate. MALDI-MS (positive): *m*/*z* 83923.6.

[35] $[\alpha]_D^{23} = -63.8\ deg\,cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.01\ g\,cm^{-3}$, H_2O); HR-ESI-MS (positive): *m*/*z* 2604.0803 $[M+H]^+$, calcd 2604.0931; ^{1}H NMR (400 MHz, D_2O, DQF-COSY): $\delta = 5.33$–5.16 (m, 4H {5.3}, H-8″, {5.27} H-4, {5.26} H-7″, {5.23} CH_{2a}-Bn-Ester), 5.00–4.86 (m, 3H, {4.95} CH_{2b}-Bn-ester, {4.91} H-4′, {4.88} H-1), 2.93 (m, 2H, S-CH_2), 2.85 (dd, 1H, $J_{gem} = 17.3$ Hz, $J_{vic} = 6.6$ Hz, D_a^{β}), 1.21–1.13 (m, 3H, T^{γ^*}), 1.06 ppm (d, 3H, $J = 6.4$ Hz, T^{γ}); ^{13}C NMR (100.6 MHz, D_2O, HSQC): $\delta = 100.8$ (C-1′), 98.9 (2C, C-1, C-2″), 36.8 (C-3″), 30.0 (SAc), 28.2 ppm (S-*C*H_2).

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[37] $[\alpha]_D^{25} = -49.4\ deg\,cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.01\ g\,cm^{-3}$, H_2O); HR-ESI-MS: *m*/*z* 2118.9705 $[M+H]^+$, calcd 2118.9760; ^{1}H NMR (400 MHz, D_2O, COSY): $\delta = 5.69$ (m, 1H, CH_2-C*H*=CH_2), 4.99 (m, 2H, CH_2-CH=CH_2), 4.80 (d, 1H, $J_{1,2} = 3.7$ Hz, H-1), 2.54 (dd, 1H, $J_{3,4} = 4.5$ Hz, $J_{gem} = 12.5$ Hz, H-3′eq.), 1.15 (d, 3H, $J = 6.2$ Hz, T^{γ^*}), 1.09–1.04 ppm (m, 6H, $2 \times T^{\gamma}$); ^{13}C NMR (100.6 MHz, D_2O, HSQC, HMBC): $\delta = 133.6$ (CH_2-*C*H=CH_2), 115.3 (CH_2-CH=*C*H_2), 99.3 (C-1), 99.2 (C-2′), 39.5 ppm (C-3′).

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